CellPress

Looking into the Black Box of Synaptic Vesicle Recruitment

Stefan Hallermann[1,*]
[1]Carl-Ludwig-Institute for Physiology, Medical Faculty, University of Leipzig, Liebigstrasse 27, 04103 Leipzig, Germany
*Correspondence: hallermann@medizin.uni-leipzig.de
http://dx.doi.org/10.1016/j.neuron.2015.10.028

To sustain ongoing synaptic transmission, new transmitter-filled vesicles must be recruited to empty release sites rapidly. However, in this issue of *Neuron*, Midorikawa and Sakaba (2015) show that, before being released, vesicles are tethered at the membrane for seconds.

To communicate with each other, neurons can use chemical synaptic transmission. Upon the arrival of an action potential, calcium influx at the presynaptic active zone triggers the fusion of transmitter-filled vesicles (Südhof, 2012). After the fusion of a subset of the release-ready vesicles (Neher, 2015), new vesicles are recruited to the active zone to sustain ongoing synaptic transmission. Our understanding of the mechanisms of vesicle recruitment is surprisingly limited, because most techniques (e.g., capacitance measurements, FM dyes, synaptopHluorins) measure exo- and endocytosis but are "blind" to the process of vesicle recruitment preceding vesicle fusion. In contrast, total internal reflection fluorescence microscopy (TIRFM) offers the chance to look into this black box of vesicle recruitment. TIRFM relies on the fact that oblique light is totally reflected, and in addition generates a very thin evanescent field that excites fluorophores within ~100 nm from the glass surface. However, to investigate the steps preceding vesicle fusion with TIRFM, active zones must be very close to the glass surface facing toward it. This technique was established at neuroendocrine cells and specialized neurons such as cones and bipolar cells (Chen et al., 2013; Zenisek et al., 2000). Yet, so far TIRFM has not been used to analyze active zones of conventional neurons, which fire action potentials.

In this issue of *Neuron*, Midorikawa and Sakaba succeeded in "replacing" the postsynaptic neurons of calyces of Held—large presynaptic terminals in the auditory brain stem—with glass coverslips (Midorikawa and Sakaba, 2015; Figure 1A). The presynaptic neurotransmitter-releasing surface, which normally faces the postsynaptic neuron, is thus directed toward the coverslip. This approach enabled the authors to visualize single vesicles near the presynaptic plasma membrane in the TIRF-field. Thereby, Midorikawa and Sakaba were able to investigate the steps preceding the fusion of a synaptic vesicle with high spatial and temporal resolution. Previous studies analyzing vesicle dynamics with superresolution microscopic techniques could not establish the exact location of vesicles with respect to the plasma membrane (Maschi and Klyachko, 2015). Analyzing the calyx of Held synapse with TIRFM is particularly exciting because the vesicle dynamics have been extensively studied with high-resolution electrophysiological techniques at this synapse (Borst and Soria van Hoeve, 2012). Thus, the study by Midorikawa and Sakaba challenges our current understanding of vesicular release with a novel and complementary approach.

The authors sparsely labeled synaptic vesicles in the slice preparation with FM dye and then acutely dissociated and plated the calyces onto coverslips. First, they focused on vesicular release elicited by calcium influx through calcium channels. They analyzed vesicle dynamics before, during, and after a 100 ms opening of calcium channels elicited by depolarization using presynaptic voltage clamp recordings. Due to the sparse FM labeling of vesicles, the detected signals represent individual vesicles. The fluorescence time course of single vesicles was measured within a circle centered on the vesicle and a concentric annulus surrounding the vesicle. Fluorescence signals of the center and annulus were classified into four groups:

(1) "Diffuse" events showed a delayed fluorescent peak at the annulus as the fluorescence at the center diminished, indicating spreading of the dye into the surrounding area upon exocytosis.

(2) "Vanish" events exhibited a diminishing time course similar to that of the diffuse events, but spreading of the dye was not detected. Since "diffuse" and "vanish" events were highly synchronized with the stimulus and occurred preferentially at calcium entry sites (defined by TIRFM-calcium imaging), both events were grouped together as "responder" events and interpreted as exocytosis.

(3) "Bounce" events showed a transient fluorescence increase with a dwell time of ~90 ms. They were accompanied by a small increase in the fluorescence of the annulus. The annulus fluorescence was synchronous with the center fluorescence and not delayed as in the "diffuse" events. Furthermore, "bounce" events were not correlated with the stimulus, arguing that "bounce" events represent neither classical exocytosis nor kiss-and-run events, but rather appearance and disappearance of vesicles at the membrane.

(4) "Tethered" events showed a sudden increase in the fluorescence that stayed constant until the end of a given recording period (~1 s). These events occurred preferentially after the onset of the stimulus,

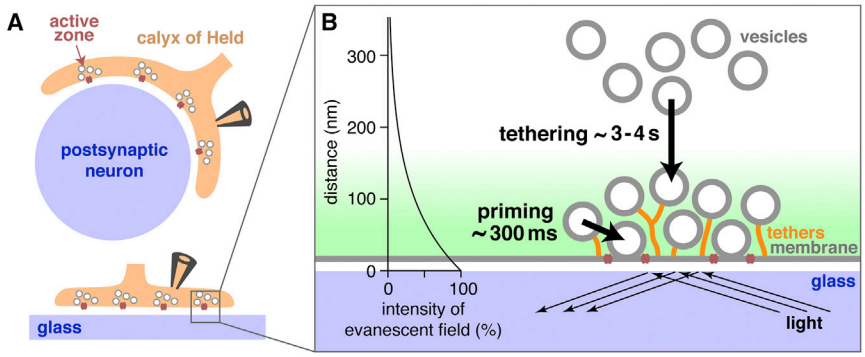

Figure 1. TIRFM at Conventional Active Zones

(A) Illustration of a calyx of Held (brown), with four active zones (red) signaling to the postsynaptic neuron (blue). Below, a dissociated calyx is illustrated with active zones directed toward the glass coverslip (blue). This allows visualizing the movement and fusion of individual FM-dye-labeled synaptic vesicles with TIRFM at an inverted microscope. Presynaptic patch clamp pipettes are illustrated.

(B) Illustration of the penetration depth of the evanescent field of the totally reflected light, assuming a decay length constant of 100 nm of the evanescent field. By combining TIRFM and advanced electrophysiological techniques, Midorikawa and Sakaba (2015) observed that tethering of vesicles (tethers illustrated in orange) was surprisingly slow (~3–4 s; room temperature) and that only those vesicles, which are tethered for seconds, can be converted to release-ready vesicles via a priming step with faster kinetics once release sites near calcium channels (red) become available (~300 ms).

suggesting that they represent the tethering of synaptic vesicles at empty release sites. These vesicles have also been referred to as "newcomer" vesicles in previous studies.

The experiments with 100 ms depolarizations already revealed the main conclusion of this study: relating the time course of newly tethered vesicles ("newcomers") to the number of released vesicles during the stimulation ("responder" events) demonstrated that tethering of new vesicles could refill the empty release sites with a time constant of ~4 s (Figure 1B). This is surprising, since the full process of vesicle tethering, docking, and priming is generally believed to be rate-limited by tethering and to happen within ~300 ms (see references in Midorikawa and Sakaba, 2015). Furthermore, at some ribbon-type synapses, "newcomer" vesicles have been suggested to be released rapidly (Chen et al., 2013; Zenisek et al., 2000), but Midorikawa and Sakaba did not observe a single "newcomer" vesicle fuse within the first second after the stimulus at conventional active zones of the calyx of Held.

The authors went on to corroborate this finding by analyzing the calcium dependence of vesicle dynamics. They repeated the 100 ms depolarization experiments with increased presynaptic calcium buffering (from 0.5 to 5 mM EGTA in the presynaptic solution). The rate constant of tethering was similar in both conditions. This is again surprising, because vesicle recruitment has been shown to be calcium dependent at several synapses (see references in Midorikawa and Sakaba, 2015). Specifically, these experiments indicate that the rapid recovery from synaptic depression, which is EGTA sensitive at the calyx, is not mediated by tethering of vesicles.

Finally, the authors performed the heroic experiments of combining single-vesicle analysis using TIRFM with calcium uncaging, which elevates intracellular calcium concentration rapidly and homogeneously. They found a time constant for tethering of ~3 s, which is comparable to the ~4 s observed with depolarization experiments. In the uncaging experiments, Midorikawa and Sakaba could also analyze a sustained release component previously described at the calyx of Held with a time constant of ~300 ms. Interestingly, "newcomer" vesicles did not participate in this sustained component. In contrast, those vesicles that were stationary but not immediately released upon calcium elevation were responsible for this sustained release component. The fact that a subset of vesicles is refractory to release despite high calcium concentrations represents yet another important finding of this study. Thus, these results suggest that the recruitment of new release-ready vesicles is mediated by conversion of tethered vesicles from nonreleasable to releasable. This process could be considered as a priming step occurring on a timescale of ~300 ms (Figure 1B).

The study by Midorikawa and Sakaba (2015) changes our current understanding of synaptic transmission by demonstrating that the recruitment of release-ready vesicles is mainly mediated by vesicles that are already tethered near the membrane for several seconds. The slow recruitment of tethered vesicles suggests that the vesicles released during high-frequency synaptic transmission must have been already tethered at the membrane. However, during high-frequency synaptic transmission, the calyx of Held releases ~20,000 vesicles within 4 s (e.g., quantal content of ~100 at 50 Hz transmission; Grande and Wang, 2011), yet based on electron microscopic studies only 1,000–3,000 vesicles are docked at the plasma membrane (Rollenhagen and Lübke, 2006). A possible explanation for this discrepancy could be that TIRFM might visualize not only docked vesicles but also vesicles in the second and third rows. Indeed, the penetration depth of the TIRF field is difficult to measure, but the decay length constant of the evanescent field seems to be in the range of 100 nm (Steyer and Almers, 1999). This would provide enough vesicles within the TIRF field, from which new vesicles can be recruited during high-frequency transmission (~45,000 vesicles within <200 nm distance from the membrane; Rollenhagen and Lübke, 2006). Indeed, the authors occasionally detected an increase in the vesicle fluorescence before fusion, which is consistent with a movement of vesicles toward the membrane. Thus, the authors suggest the presence of a large pool of tethered vesicles (Saviane and Silver, 2006), which feeds the vesicles to the release sites through priming. It is conceivable that longer tethers (including actin filaments) mediate the initial tethering (Hallermann and Silver, 2013) and shorter tethers (including SNARE proteins) mediate the subsequent priming (Imig et al., 2014).

In some of the previous studies performed at the calyx as well as other synapses, faster recruitment of release-ready

vesicles has been reported in the range of 30 ms (Kushmerick et al., 2006; Ritzau-Jost et al., 2014; Saviane and Silver, 2006). Midorikawa and Sakaba point out that their TIRFM experiments were performed in immature animals and at room temperature. Furthermore, the enzymatic dissociation of the calyx from the postsynaptic neuron could slow down vesicle recruitment processes. However, the authors demonstrate that all of the investigated functional parameters in dissociated calyces were similar to previous studies on intact calyces.

In summary, the combination of presynaptic recordings, calcium uncaging, calcium imaging, and high-resolution visualization of single vesicles with TIRFM at conventional active zones represents a technical breakthrough which will serve as a benchmark for future studies. This allowed Midorikawa and Sakaba to look into the black box of vesicle recruitment, where they found that vesicles are tethered for several seconds before being released.

REFERENCES

Borst, J.G., and Soria van Hoeve, J. (2012). Annu. Rev. Physiol. *74*, 199–224.

Chen, M., Van Hook, M.J., Zenisek, D., and Thoreson, W.B. (2013). J. Neurosci. *33*, 2071–2086.

Grande, G., and Wang, L.Y. (2011). J. Neurosci. *31*, 13386–13399.

Hallermann, S., and Silver, R.A. (2013). Trends Neurosci. *36*, 185–194.

Imig, C., Min, S.W., Krinner, S., Arancillo, M., Rosenmund, C., Südhof, T.C., Rhee, J., Brose, N., and Cooper, B.H. (2014). Neuron *84*, 416–431.

Kushmerick, C., Renden, R., and von Gersdorff, H. (2006). J. Neurosci. *26*, 1366–1377.

Maschi, D., and Klyachko, V.A. (2015). Synapse *69*, 256–267.

Midorikawa, M., and Sakaba, T. (2015). Neuron *88*, this issue, 492–498.

Neher, E. (2015). Neuron *87*, 1131–1142.

Ritzau-Jost, A., Delvendahl, I., Rings, A., Byczkowicz, N., Harada, H., Shigemoto, R., Hirrlinger, J., Eilers, J., and Hallermann, S. (2014). Neuron *84*, 152–163.

Rollenhagen, A., and Lübke, J.H.R. (2006). Cell Tissue Res. *326*, 221–237.

Saviane, C., and Silver, R.A. (2006). Nature *439*, 983–987.

Steyer, J.A., and Almers, W. (1999). Biophys. J. *76*, 2262–2271.

Südhof, T.C. (2012). Neuron *75*, 11–25.

Zenisek, D., Steyer, J.A., and Almers, W. (2000). Nature *406*, 849–854.

Sensory-Motor Circuits: *Hox* Genes Get in Touch

Polyxeni Philippidou[1] **and Jeremy S. Dasen**[1,*]
[1]Neuroscience Institute, Department of Neuroscience and Physiology, NYU School of Medicine, New York, NY 10016, USA
*Correspondence: jeremy.dasen@nyumc.org
http://dx.doi.org/10.1016/j.neuron.2015.10.035

Sensory-motor reflex circuits are the basic units from which animal nervous systems are constructed, yet little is known regarding how connections within these simple networks are established. In papers in *Cell Reports* and in this issue of *Neuron*, Zheng et al. (2015a, 2015b) demonstrate that coordinate activities of *Hox* genes in sensory neurons and interneurons govern connectivity within touch-reflex circuits in *C. elegans*.

The diversity of animal behaviors relies on the ability of neurons to establish selective synaptic connections with target cells and integrate into functionally distinct networks. This process is facilitated by genetic programs that define the unique molecular features of individual neurons within a circuit. For example, retinal ganglion cells acquire subtype-specific properties that are necessary within circuits that encode for varied aspects of visual stimuli (Sanes and Masland, 2015). In the spinal cord, motor neurons undergo diversification programs that enable them to selectively target peripheral muscles and engage with local and supraspinal networks involved in walking and breathing (Catela et al., 2015). Resolving the genetic mechanisms underlying connectivity within sensory and motor systems in vertebrates has been challenging, due in part to the large number of neuronal subtypes involved, and the complexity of their interconnections.

The relatively simple nervous system of the nematode *C. elegans* has been instrumental in elucidating the basic programs underlying neuronal subtype specification and connectivity. The lineage, morphology, and synaptic partners of each of the 302 neurons in *C.elegans* are well characterized, and many key neuronal fate determinants have been identified (Hobert, 2010). In *C. elegans*, distinct sensory-motor circuits mediate forward and backward escape movements in response to posterior and anterior light body touch, respectively. While the wiring diagrams for these circuits have been described (Schafer, 2015), the rules that govern their assembly are not well understood.

In *C. elegans* six touch receptor sensory neurons (TRNs) mediate responses to light touch. TRNs diversify into four subtypes, including two bilaterally symmetrical pairs that arise during embryogenesis: anterior ALM and posterior PLM neurons. ALM neurons mediate backward movement in response to sensory stimuli, while PLM neurons facilitate